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The new medium MDSS2N, free of any animal protein supports cell growth and production of various viruses

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Abstract

The development of media free of serum and animal or human proteins is of utmost importance for increasing the safety of biologicals produced for therapy and vaccination. In order to reduce the risk of contamination, we have modified the serum free medium MDSS2, a very efficient serum free medium for the production of various biologicals including experimental vaccines using different cell lines (Merten et al., 1994), by replacing the animal derived products by plant extracts. The new serum and animal protein free medium (MDSS2N) can be efficiently used for biomass production of various cell lines. These cells grow equally well or better in this new serum-free medium than in the old formulation (MDSS2):

- BHK-21/BRS cells, adapted to MDSS2N, showed an overall specific growth rate of 0.0197 h^{-1} (μ_{max} = 0.0510±0.0058 h^{-1}), whereas those cultivated in MDSS2 grew with an average specific growth rate of 0.0179 h^{-1} (μ_{max} = 0.0305±0.0177 h^{-1}).
- Vero cells grew with an average specific growth rate of 0.0159 h^{-1} and 0.0153 h^{-1} in MDSS2 and MDSS2N, respectively. Very similar growth rates were obtained in microcarrier cultures in stirred tank reactors: the specific growth rates were 0.0161 h^{-1} and 0.0166 h^{-1} for MDSS2 and MDSS2N cultures, respectively.
- For MDCK cells, when cultured on microcarriers in bioreactors, a higher average specific growth rate was observed in MDSS2N than in MDSS2; values of 0.0248 h^{-1} and 0.0168 h^{-1}, respectively, were obtained.

The capacity of MDSS2N to support the production of different viruses was equally evaluated and it could be established that for certain viruses there are no or insignificant differences between MDSS2N and MDSS2 (influenza and polio virus), whereas, the production of rabies virus is somewhat reduced in MDSS2N when compared to MDSS2. The use of MDSS2N for cell culture and the production of various viruses is discussed.

Introduction

Although the supplementation of cell culture media with various animal sera has several advantages and facilitates generally animal cell technology, the use of serum presents several disadvantages. The main drawbacks are: 1. the introduction of animal derived proteins, leading to the necessity to eliminate these

proteins during downstream processing; 2. the potential introduction of microbial contaminants, like fungi, bacteria, viruses or the BSE-agent; 3. the variable quality and high costs. Amongst these drawbacks, the potential introduction of contaminants (adventitous agents) is the most problematic issue, leading to the extended use of serum-free media (SFM). However, most of the current SFM are based on the use of animal derived peptones and hydrolysates (Jan et al., 1994; Keay, 1975, 1977, 1978; Litwin, 1991, 1992; Merten et al., 1994; Schlaeger et al., 1993; Schlaeger 1996) and/or animal derived purified proteins (Jäger et al., 1988; Shacter, 1987). Although this development is a step further in the right direction this is not sufficient because the potential risk of introducing adventitious agents is still present. Therefore, the aim should be the development of a serum-free medium which is free of any animal derived product.

Several years ago, we have developed the SFM MDSS2 (Merten et al., 1994), which could be used for cell growth and the production of various biologicals: e.g. rec. BHK cells for the production of rec. IL-2 (Merten et al., 1994), BHK-21 cells for the production of rabies virus (Perrin et al., 1995), Vero cells for the production of polio virus (Merten et al., 1997), and MDCK cells for the production of influenza virus (Merten et al., 1996)). Despite the large potential of this SFM, it was not accepted by industry, mainly because of the presence of casein peptone, and although milk and milk products are at very low risk with respect to BSE-transmission (category IV) (Committee for Proprietary Medicinal Products. EEC Regulatory Document. Note for Guidance 1992). We, therefore, decided to continue the development of this SFM with the aim to replace the animal derived peptone by plant extracts.

In this work we present the evaluation of the modified SFM MDSS2N, in which casein peptone has been replaced by a soy-bean derived peptone, for the cell growth of BHK-21, Vero, and MDCK cells. In addition, we have evaluated the ability of MDSS2N to sustain the production of various viruses. In this context, results obtained from rabies, polio as well as influenza virus productions are presented.

Materials and methods

Cell lines: Vero cells (WHO-1878) were obtained in the 137th passage from Dr F. Horaud (Institut Pasteur, Unité de Virologie Virale). These cells were adapted to growth in the SFM MDSS2 (Merten et al., 1994) and were stored in liquid nitrogen in the SFM MDSS2 (Merten et al., 1995) at passage 167. The cells were routinely passed once a week in DMEM supplemented with 5% fetal calf serum (FCS) or in MDSS2 or in MDSS2N. BHK-21/BRS cells were derived from BHK-21 C13 and adapted to suspension growth in MDSS2 (Perrin et al. (1995)). These cells were routinely passed twice a week in MDSS2 and in MDSS2N. MDCK cells (originally obtained from NIH (Bethesda, USA)) were adapted to growth in SFM, stored in liquid nitrogen in the SFM MDSS2 (Merten et al., 1995), and generally passed once a week in MDSS2 or in MDSS2N.

Virus strains: The rabies virus PV-Paris/BHK-21 and PV-Paris/BHK-21/BRS were used to infect the BHK-21/BRS cells (Perrin et al. (1995)).

The polio virus strain Sabin 1 (SI $(S_{0+1})_{+2}$, frozen on the 9th of December 1995; titer $7x10^7$ PFU/ml) was used to infect the Vero cells.

The influenza virus strain A/Texas/36/91(H1N1) was used to infect the MDCK cells (Merten et al., 1998).

Culture Media: DMEM (Axcell Biotechnologies 36213 SPM) + 5% FCS (Hyclone A-1115-L), MDSS2 (Axcell Biotechnologies, St. Genis l'Argentière/F, n° 34601) and MDSS2N (commercialized under the name AXCEVIR-Vero^{TM} by Axcell Biotechnologies, n° 34603) were used.

Growth assays: Cell culture assays were performed in 10 ml of culture medium at 37 °C, in 25 cm^2 flasks, at an initial concentration ranging between 0.5×10^5 and 1×10^5 cells/ml for BHK-21/BRS cells. For Vero cells, 25 cm^2 flasks were inoculated with 2×10^4 cells/cm^2. Samples were taken daily to determine the cell concentration. The assays were performed in duplicate. Generally, three passages were performed, as described by Merten et al. (1994).

Bioreactor cultures: The cultures were performed in a 2 litres Biolafitte ICC 1L and an Inceltech LH Series 210 reactor (1.6 l working volume) equipped with a spin filter (pore size: 18 μm) fixed on the axes for the retention of cells growing on microcarriers. The following conditions were applied: pH = 7.2, pO_2 = 20% air-saturation, temperature = 36.5–37 °C and agitation = 45 rpm (Inceltech) or 50 rpm (Biolafitte).

Cells were grown on microcarriers: Superbead

(Flow 60-085-12) or Cytodex 1 (Pharmacia 17-0448-01) at a concentration of 2.5 g/l, 5 g/l and 6.25 g/l. The cultures were seeded with 1–2$\times 10^5$ cells per ml in the presence of microcarriers. To initiate the cultures, the cell/microcarrier suspension was agitated discontinuously for 5 min with an interruption of 10 min for 12 h and then agitated continuously at 45–50 rpm. All cultures, except one reactor culture of Vero cells (see Figure 3), were performed in perfusion mode. Perfusion was started when the cell density was about 4.5–7 $\times$ 10^5 cells/ml and the perfusion rate was adjusted with respect to the cell density.

Cell counting: For BHK-21/BRS cells growing in clumps, 0.5 ml of a trypsin/versene solution (0.1% trypsin-solution in PBS: 0.04% versene-solution in PBS, v/v) was added to 0.5 ml of the cell suspension. After incubation at 37 °C for 10 minutes, cells were stained with trypan blue (0.2% in PBS) and counted. Vero cells were washed with PBS then trypsinized at 37 °C for 5 to 10 minutes and counted. For counting Vero and MDCK cells grown on microcarriers, a 0.5ml sample was treated with 0.5 ml 0.1M citric acid containing 0.1% crystal violet and 0.1% Triton X-100 and incubated at 37 °C for at least one hour, the released cell nuclei were counted.

Virus production: Rabies virus: BHK-21/BRS cells were infected by two strains of rabies virus (PV-Paris/BHK-21 and PV-Paris/BHK-21/BRS) at a cell concentration of 4 $\times$ 10^6 cells/ml with a MOI of 0.1. Virus production was performed at 34 °C, in 50 ml agitated tubes containing 10 ml of the medium to be tested. Samples were taken daily to determine cell concentration, virus titre and glycoprotein content. The assays were performed in duplicate.

Polio virus: Six well plates (10cm^2/well) were inoculated with 26.6$\times 10^3$ cells/cm^2 and cultivated in the media to be compared. Seven days later, the medium was eliminated, the cells were washed twice with 2ml PBS per well and the cell layer was incubated with polio virus (strain Sabin I) at a MOI of 10 for about 45 min (volume: about 500 μl). Then 3 ml/well of culture medium, containing 50μg/ml gentamycin, was added. The assays were performed in duplicate. Two days later all cells were dead, the supernatant was recovered, once centrifuged (3000 rpm, 10min, 4 °C), and frozen at –20 °C.

Influenza virus: For infecting the reactor cultures, the perfusion was discontinued, and virus was added at a MOI of about 0.01. After 45–60 min after addition of the virus, trypsin TPCK, arginin and gentamycin were added at the following concentrations: 2 μg/ml, 270 mg/l, and 50 μg/ml, respectively. At the same time the residual glucose concentration of the culture medium was increased by 3 g/l. The perfusion was restarted, using the same medium as for cell culture, supplemented with trypsin TPCK, arginin, and gentamycin at the above mentioned concentrations, and which contained a doubled glucose concentration.

Rabies virus titration: Virus titres were determined according to a modified RFFIT method (Smith et al. (1973)) and expressed in Fluorescent Focus Units per ml (FFU/ml).

Rabies glycoprotein titration: rabies glycoprotein content was determined by ELISA using polyclonal antibodies according to Perrin et al. (1990).

Polio virus titration: The virus titration was done by using the dilution method according to the WHO (1990), whereby six parallel microcultures in 96 well plates were infected per virus (sample) dilution. The titers were expressed as PFU/ml.

Influenza virus: Virus titers were measured by the standard hemagglutination test using guinea pig red blood cells and given in HA units (HAU).

Results

Cell growth in animal protein-free medium

BHK-21/BRS cells:
MDSS2N was compared to MDSS2 for supporting the growth of BHK-21/BRS cells. Tests performed in T-flasks showed that the maximal cell density of BHK-21/BRS cells obtained was 1.3$\times 10^6$ cells/ml and 1.1$\times 10^6$ cells/ml, for MDSS2N and MDSS2, respectively (Figure 1). The average specific growth rates were 0.0197 (μ_{max} = 0.0510$\pm$0.0058 h^{-1}) and 0.0179 h^{-1} (μ_{max} = 0.0305$\pm$0.0177 h^{-1}) in MDSS2N and MDSS2, respectively. These results indicate that BHK-21/BRS cells exhibit rather similar growth kinetics in the new protein-free medium MDSS2N as in the previously developed medium MDSS2.

Vero cells:
MDSS2N was also evaluated for its ability to sustain the growth of Vero cells in static (T-flasks) and agitated (microcarrier in bioreactor) cultures.

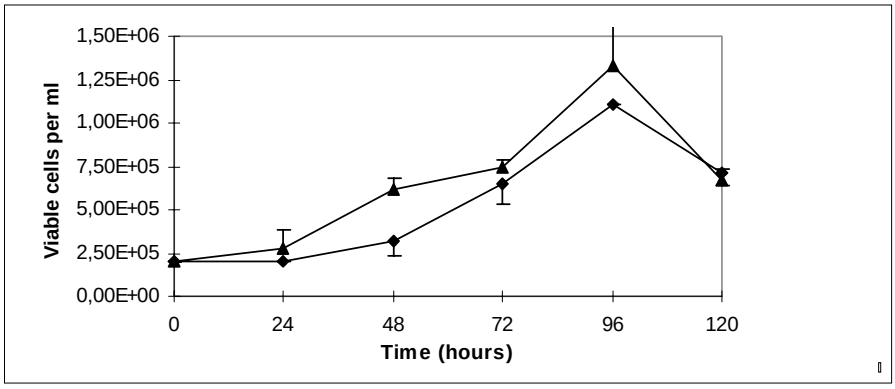

Figure 1. Growth of BHK-21/BRS cells in T-flasks (25cm^2) in MDSS2N (triangles) and MDSS2 (diamonds); the error bars are indicated (n=2).

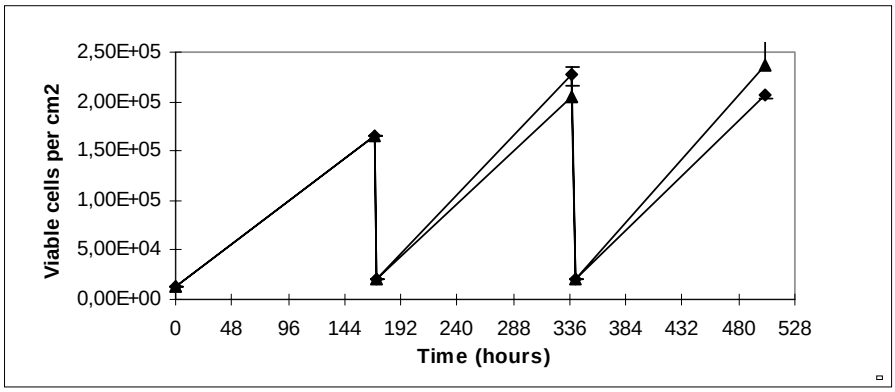

Figure 2. Growth of Vero cells in T-flasks (25cm^2) in MDSS2N (triangles) and MDSS2 (diamonds). At 168 h and 336 h the cultures were passaged, the subcultures were started at 20000 c/cm^2; the error bars are indicated (n=2).

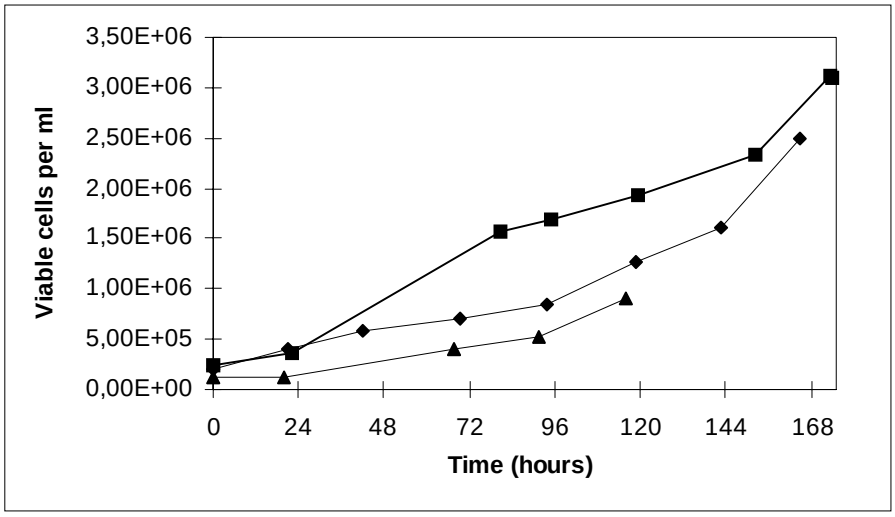

Figure 3a.

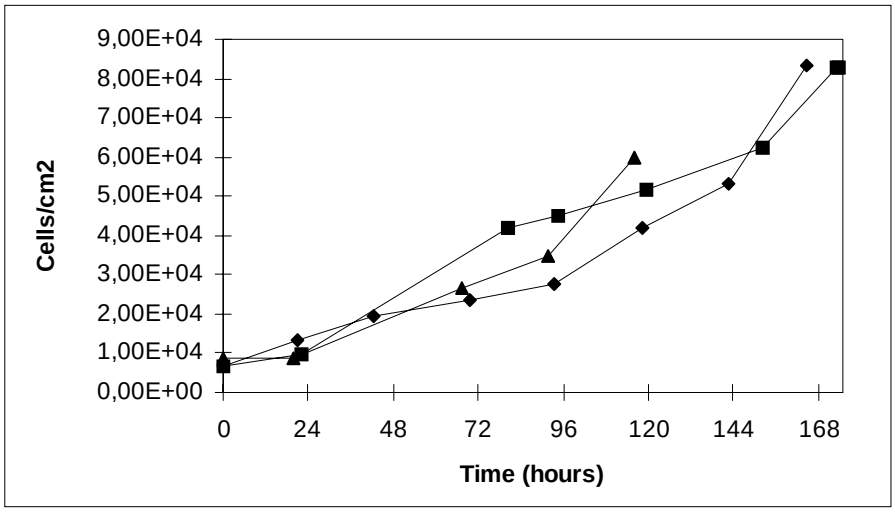

Figure 3b. Growth of Vero cells in a 1.6 L bioreactor in MDSS2N (on 2.5 g/l Cytodex I, triangles), in MDSS2 (on 5 g/l Cytodex I, diamonds) and in DMEM supplemented with 5% FCS (on 6.25 g/l Superbeads, squares). The cell densities per ml of culture (A) as well as the cell density per cm^2 of carrier (B) are shown.

Table 1. Average specific growth rates of Vero cells grown on microcarriers in stirred tank reactors in MDSS2N, MDSS2, or DMEM + 5% FCS (see Figure 3)

	MDSS2N	MDSS2	DMEM + 5% FCS
Average specific growth rate (1/h)	0.0166	0.0161	0.0147

After 3 successive static subcultures of Vero cells in MDSS2N and MDSS2, a maximum cell density was 2.36×10^5 and 2.08×10^5 cells/cm^2 was reached in MDSS2N and MDSS2, respectively (Figure 2). The average specific growth rate was 0.0153 h^{-1} (μ_{max} = 0.026±0 h^{-1}) and 0.0159 h^{-1}(μ_{max} = 0.027±0.001 h^{-1}), in MDSS2N and MDSS2, respectively. These results show that Vero cells grow equally well in MDSS2N and MDSS2. For the reactor cultures different cell densities were obtained, when three different media were used. The maximal cell densities were 3.1×10^6 c/ml, 2.5×10^6 c/ml, and 0.897×10^6 c/ml when DMEM + 5% FCS, MDSS2, and MDSS2N, respectively, were used (Figure 3A). These differences were essentially due to the concentration of the microcarriers used. Indeed, when comparing the average specific growth rates for these three cultures, the cultures in both serum-free media were found to have a slightly higher growth rate than the culture in DMEM + 5% FCS (Table 1). In addition, comparison of the cell density expressed as the number of cells per cm^2 of microcarriers, clearly shows that for the three media used the increase in the cell number per cm^2 was comparable (Figure 3B). The observation that the culture in MDSS2N stopped earlier than those in MDSS2 or DMEM + 5% FCS was due to the fact that the available surface was limited and saturated at 116 h and that unlike the other cultures this culture was conducted as a batch and not as a perfusion culture.

MDCK cells:

The growth of MDCK cells in both SFM MDSS2 and MDSS2N was evaluated for microcarrier cultures in stirred tank reactors. For the cultures presented in Figure 4, the cells started to grow after a lag phase of 24–48 hours and about 162 hours after onset cell densities of 4.2×10^6 and 5.8×10^6 c/ml were obtained in MDSS2 and MDSS2N, respectively. This signifies that the overall specific growth rate, calculated between the onset of the culture and 162 hours, was higher for the cells grown in MDSS2N (0.0248 h^{-1}) than for those grown in MDSS2 (0.0168/h). The maximal specific growth rates were 0.05 h^{-1} and 0.041 h^{-1}, respectively, for cultures in MDSS2N and MDSS2. At 162 h, the cultures were infected with influenza virus (see later for virus titers). Approximately 24 h after infection the cell densities started to decrease due to detachment of the cells from the carriers caused by the presence of trypsin during the virus production phase, but also due to the multiplication of

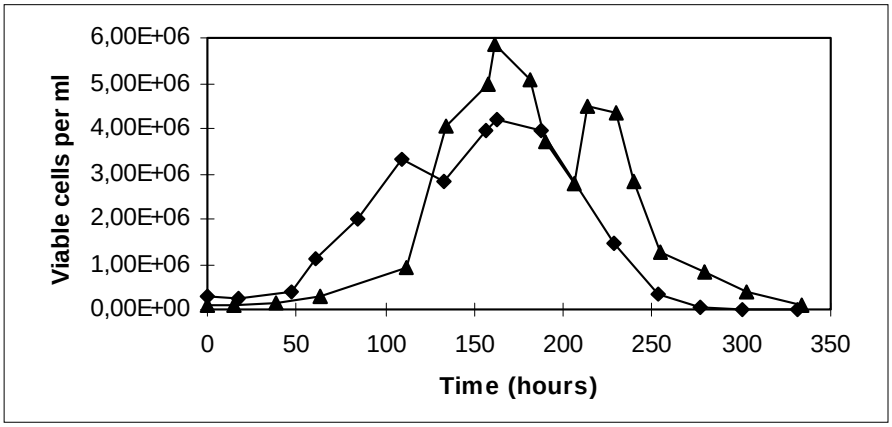

Figure 4. Comparison of cell growth for MDCK cells grown on microcarriers in MDSS2N (triangles) and MDSS2 (diamonds). MDCK cells were grown on Cytodex I (5g/l) microcarriers in a 1.6 L Inceltech LH Series 210 reactor either MDSS2N or MDSS2 at an initial cell density of 1.1×10^5 and 2.7×10^5 c/ml, respectively. At the indicated time points, cells were counted and cell densities determined. At 162 h the cells were infected with influenza virus A/Texas/36/91 (H1N1).

Table 2. Maximal rabies virus titres in FFU/ml, produced by BHK-21/BRS cells infected with PV-Paris/BHK-21 and PV-Paris/BHK-21/BRS strains, in MDSS2 and MDSS2N (n=2)

	PV-Paris/BHK-21∗∗	PV-Paris/BHK-21/BRS∗
MDSS2N	$0.77\times10^6\pm0.115\times10^6$	$1.47\times10^6\pm0.22\times10^6$
MDSS2	$1.86\times10^6\pm0.279\times10^6$	$2.37\times10^6\pm0.355\times10^6$

∗ The difference is weakly significant on a 10% level (t-test),
∗∗ The difference is significant on a 5% level (t-test)

the virus which leads to apoptotic death of the infected cells.

Production of viruses on cells grown in animal protein-free medium

Rabies virus on BHK-21/BRS cells: To evaluate the capacity of the new medium MDSS2N to support the production of rabies virus, we infected BHK-21/BRS cells (grown in MDSS2N or in MDSS2) with two strains of this virus: PV-Paris/BHK-21/BRS (a strain adapted to cells grown in MDSS2) and PV-Paris/BHK-21 (a strain non adapted to cells grown in MDSS2).

The results in Figure 5 show that in both media the behaviour of BHK-21/BRS cells infected with PV-Paris/BHK-21 or PV-Paris/BHK-21/BRS was comparable. In both cases, a decrease of the cell density from 4×10^6 cells/ml to $0.5–1\times10^6$ cells/ml, was observed 5 days post infection.

After infection with the strain PV-Paris/BHK-21 a maximal virus titre of $0.77\times10^6\pm0.115\times10^6$ FFU/ml was obtained in MDSS2N. In MDSS2, virus titres were 2.4 times higher ($P<0.05$)(Table 2). After infection with the adapted PV-Paris/BHK-21/BRS strain, titers of $1.47\times10^6\pm0.22\times10^6$ FFU/ml and $2.37\times10^6\pm0.355\times10^6$ FFU/ml, respectively, were obtained in MDSS2N and MDSS2.

Figure 5 also shows that when cells were infected with the non adapted strain (PV-Paris/BHK-21), the levels of glycoprotein produced at the end of the culture, were lower in MDSS2N (338.3 ± 67.7 ng/ml) than in MDSS2 (927.8 ± 185.6 ng/ml)($P<0.02$). The specific productivity of glycoprotein was also lower in MDSS2N than in MDSS2 (Table 3).

After the infection with PV-Paris/BHK-21/BRS strain the overall levels of glycoprotein production were higher in MDSS2 than in MDSS2N, respectively, 3596 ± 719 and 1707 ± 341 ng/ml ($P<0.10$), (see Figure 5).

Specific productivity of glycoprotein in MDSS2N was 4.30 ± 0.86 $\mu g/10^7$ cells whereas that in MDSS2 was 9.25 ± 1.85 $\mu g/10^7$ cells (see Table 3). These values are comparable to those obtained when using the standard roller process for which the productivity ranged from 8 to 12 $\mu g/10^7$ cells and to those obtained by Merten et al. (1994).

Comparison of the 'immune' glycoprotein levels obtained in the different conditions (see Table 3) shows that the percentage of 'immune' glycoprotein was slightly higher after infection with PV-Paris/BHK-21/BRS in MDSS2N or MDSS2, than after infection with PV-Paris/BHK-21.

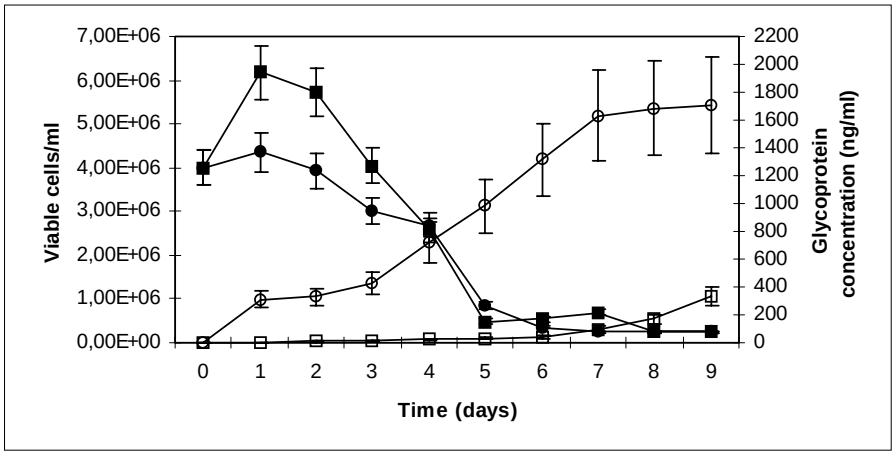

Figure 5a.

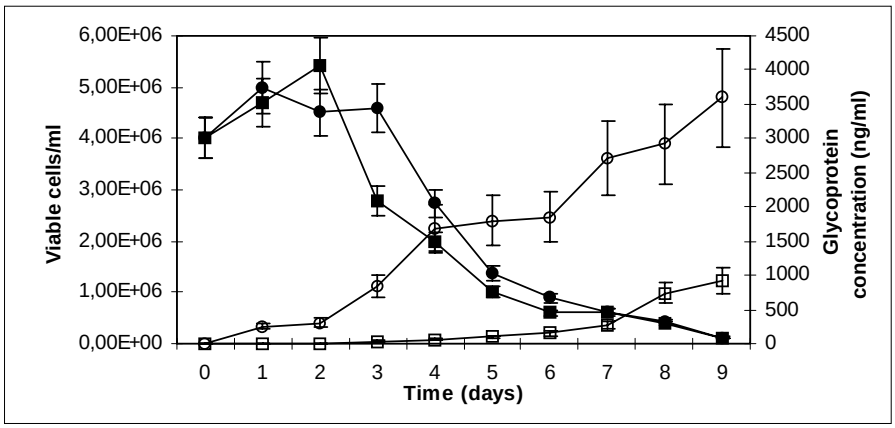

Figure 5b. Comparison of the kinetics of rabies virus glycoprotein production by BHK-21/BRS cells in MDSS2N or MDSS2: BHK21 cells grown either in MDSS2N (A) or MDSS2 (B) were infected with PV-Paris/BHK-21 (squares) or PV-Paris/BHK-21/BRS (circles). Each day the cell densities (closed symbols) as well as the glycoprotein production (open symbols) were determined; the error bars are indicated (n=2).

Table 3. Specific productivity of viral glycoprotein by BHK-21/BRS cells and level of the 'immune' glycoprotein obtained in MDSS2N and MDSS2 (n=2)

	PV-Paris/BHK-21		PV-Paris/BHK-21/BRS	
	Specific productivity of glycoprotein (μg/10^7 cells)[a]	'immune' glycoprotein (%)[b]	Specific productivity of glycoprotein (μg/10^7 cells)[a]	'immune' glycoprotein (%)[b]
MDSS2N	0.85±0.17	83.8±16.76	4.30±0.86	86.3±17.26
MDSS2	2.30±0.46	90.02±18.01	9.25±1.85	96.3±19.26

[a] The values correspond to the ratio of final glycoprotein concentration (μg)/cell density before infection $\times$ 10^7. The differences between the specific productivities are weekly significant on 10% level (t-test).

[b] The 'immune' glycoprotein level was calculated by subtracting 'the soluble' glycoprotein content from total glycoprotein. The soluble glycoprotein content was measured in the supernatant after centrifugation of the medium at the end of the culture, at 40 000 rpm at 4 °C and for 2 hours. The glycoprotein content was determined by ELISA as described in Materials and methods.

Table 4. Comparison of three media for the production of poliovirus

	DMEM + 2% FCS	MDSS2	MDSS2N
Cell density at virus inoculation $\times 10^{-6}$ c/10cm^2	4.338 ± 0.548	2.988 ± 0.018	2.850 ± 0.141
PFU/ml $\times 10^{-8}$	6.32 ± 0	4.16 ± 3.055	3.635 ± 1.054
Specific virus production PFU/c	437.07	417.66	382.62

Virus titers were determined by the limiting dilution method two days after virus inocluation as described in Materials and methods.

Poliovirus on Vero cells:

The potential of virus production of Vero cells cultivated in both SFM and in DMEM supplemented with 2% FCS was evaluated by infecting these cells with poliovirus. Approximately confluent cultures, representing $4.34\pm0.55\times10^6$ c/10cm^2, $2.99\pm0.02\times10^6$ c/10cm^2, and $2.85\pm0.14\times10^6$ c/10cm^2 for the cultures done in DMEM + 2% FCS, MDSS2, and MDSS2N, respectively, were infected with poliovirus (Sabin 1) at a MOI of 10. Two days later, after total cell lysis, rather similar virus titers were obtained, with values ranging from 3.6×10^8 – 6.3×10^8 PFU/ml (Table 4). The only significant difference in virus titer ($P < 0.02$) was that between the cells grown in DMEM+2%FCS ($6.3\times10^8\pm0$ PFU/ml) and those grown in MDSS2N ($3.6\pm1.05\times10^8$ PFU/ml). When comparing the specific virus production over a duration of 48 h (Table 4) it was found that the cells grown in MDSS2N showed a slightly reduced production rate, which was about 8.4% less with respect to cells grown in MDSS2.

Influenza virus on MDCK cells:

For evaluation of influenza virus production by MDCK cells in the new SFM, perfusion cultures were used. At 162 h, cultures in MDSS2N and MDSS2 (see Figure 4) were infected with influenza virus (A/Texas/36/91(H1N1)) at a MOI of about 0.01. Cells were kept in the same medium supplemented with 2μg/ml trypsin, 270 mg/ml arginine, 50 μg/ml gentamycine, and enriched with glucose as described in Materials and Methods. As shown in Figure 6, the virus production rates were comparable for both cultures, and slightly higher virus titers were obtained with the MDSS2N medium (HAU=640 at 141h P.I.) as compared to the MDSS2 medium (HAU=512 at 139h P.I.). Thus, SFM, in which the casein peptone component has been replaced by soy-bean extract, very efficiently supports the growth of MDCK in perfused microcarrier bioreactor cultures and also allows for the production of influenza virus at higher titers.

Discussion

The modification of MDSS2 is the new medium MDSS2N, which does not contain any animal derived products (proteins, peptones) any more. This is a considerable improvement, because this modification leads to a much safer SFM, which is free of any risk of appearance of adventitious agents associated with the use of animal derived substances. This corresponds to the general trend in the development of animal cell processes in industry (e.g.: Noé et al., 1994). Most of the SFM, actually used and/or published in the literature contain more or less animal derived products like meat peptones, insulin, transferrin, or albumin. The reason is that for many cell lines, these substances are essential for correct growth and hence for production of biologicals. Also, SFM were often developed for many different cell lines, thus requiring the development of a more universal and therefore more complex SFM capable of meeting the different nutritional needs of these cell lines.

Actually, most of the animal derived products can be replaced by synthetic substances or by plant derived products. Meat peptones, like Primatone, an additive for many SFM for a number of different cell lines (Jan et al., 1994, Keay, 1975, 1977, 1978, Litwin, 1991, 1992, Schlaeger et al., 1993, Schlaeger, 1996) or casein peptone (used in MDSS2) (Merten et al., 1994) can rather easily be replaced by plant derived peptones, like for instance by soy-bean hydrolysate as used by us (MDSS2N, this paper) and by Noé et al. (1994). Other animal derived proteins can be replaced by their recombinant equivalents (like for insulin) or non-animal derived substances replacing one or several functions of the protein, in the case the recombinant protein does not meet the requirements of the cell lines (like for albumin (Keenan et al., 1997)) or does not exist for the moment (like for transferrin (e.g. Schneider, 1989, Yabe et al., 1987)). Despite these possibilities, for some very special cell lines, the presence of animal derived proteins cannot be replaced

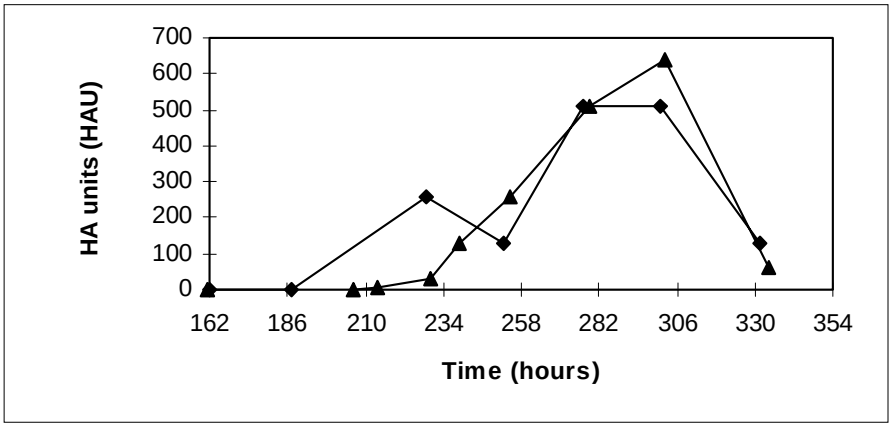

Figure 6. Comparison of influenza virus production for MDCK cells grown on microcarriers in MDSS2N or MDSS2. The MDCK cell cultures grown in MDSS2N (triangles) or MDSS2 (diamonds), presented in Figure 4, were infected at 162 h with influenza virus A/Texas/36/91(H1N1) and kept in their respective media supplemented as described in Materials and methods. At the indicated time points, virus titers were determined by a standard hemagglutination test.

by a similar recombinant protein or by a combination of several non-protein additives for the moment.

New SFM or modified SFM should provide at least a similar cell growth supporting activity as a serum-containing medium (SCM) or the previous SFM. Indeed, all cell lines included in this evaluation showed similar growth characteristics in static (T-flasks) as well as in suspension cultures (clump cultures for BHK-21 or microcarrier cultures for Vero and MDCK cells) in both SFM and in the serum-supplemented DMEM (only shown for Vero cells).

Furthermore new SFM or modified SFM should efficiently support the production of biologicals: The production of rabies virus and of viral glycoprotein by *BHK-21* cells was found to be approximately two fold lower in MDSS2N than in MDSS2. The use of virus adapted to cells grown in MDSS2 yielded significantly higher virus production ($P<0.05$) than when non adapted virus (virus adpated to cells grown in monolayers in SCM) was used. These differences may be due to the fact that the virus is not adapted to the cells grown in the new SFM MDSS2N. Several passages of the virus on the cells grown in MDSS2N might lead to a well adapted virus, as shown for the production of rabies virus by BHK-21 cells grown in MDSS2 (Perrin et al., 1995). Another reason for the differences observed, could be that the MDSS2N (although very similar to MDSS2) is to a certain extent less suitable than MDSS2 for the production of rabies virus on BHK-cells. However, it should be noted that the differences between both SFM with respect to virus production are not that dramatic, that this would preclude the use of the safer SFM MDSS2.

Many viral vaccines for human use are produced on *Vero* cells. Generally, these processes were developed about 15 years ago, and they are still serum-based reactor processes (e. g. production of polio virus on Vero cells in 1000 l reactors (Montagnon et al., 1981, 1984)). Despite the fact that the replacement of SCM by SFM is rather simple, at least for the production of polio virus (e.g. shown by us in reactor cultures done in MDSS2 (Merten et al., 1997), or also shown by Cinatl et al. (1993) in plate cultures by using the SFM PFEK-1, a very complex protein-free medium), the industry is still using SCM for these processes. Although we did not evaluate the production of polio virus in reactor cultures for Vero cells in MDSS2N, the results obtained with cultures performed in 6 well plates indicated a more or less similar virus titer in MDSS2N as compared to MDSS2. The relatively small difference between both SFM and the somewhat larger difference of the titers between SFM and SCM cultures can probably be attributed to differences in cell physiology/cell metabolism caused by the different medium composition. Such differences in virus titer can be avoided by medium optimisation as shown in reactor cultures of Vero cells for the production of polio virus (Merten et al., 1997). Although it is likely that industry is working on the development of protein-free media SFM for the cultivation of Vero cells, no information about such media is presently available, thus rendering comparison with other media difficult.

The production of influenza virus for vaccine use is still done in embryonated eggs, although companies, like Solvay Duphar (The Netherlands) or Biochem Vaccines (Canada), are already in Phase III clinical trials of a vaccine produced in serum-free *MDCK* cultures. MDSS2N can easily replace MDSS2 for the production of influenza virus on MDCK cells, leading generally to the same infectious virus yield (Merten et al., 1998) and the same HA titers (this publication, Merten et al., 1998). This is already a rather important success because the SFM MDSS2N is the only medium which is completely free of animal derived proteins. Other media which were used for the production of influenza virus, such as the EpiSerf (Gibco) (Brands et al., 1997) or Ultra-MDCK (BioWhittaker) (Kessler et al., 1998) still contain animal derived proteins, however, their exact formulations are not available. The main difference between our results and data reported by Kessler et al. (1988), is the rather important difference between virus titers, which is probably due to the MDCK clones used (age of culture, origin of clones, passage history), the composition of the media and the virus strains used. In preliminary comparative tests, we observed that cell growth of MDCK cells and virus production (A/Texas/36/91 (H1N1)) were comparable in MDSS2N and Ultra-MDCK (unpublished results) indicating that the differences between the MDCK clones used by us and Kessler et al. (1998) are probably the cause of the great differences in the virus titers.

The only animal derived product in the medium MDSS2N used for influenza virus production is trypsin which is necessary for the maturation of the virus. There are two potential solutions to circumvent this problem: 1) trypsin might be replaced by plant derived proteases hydrolyzing at the same site within the hemagglutinin as trypsin (for passing cells papain can be used for cell detachment (unpublished results)), or 2) as was shown by Kessler et al. (1998), virus strains can be adapted to grow in the absence of any exogeneous proteolytic activity to comparable virus titers as in the presence of trypsin. However, this latter approach is only applicable when the duration of adaptation is not too long because the vaccine strains have to be produced rapidly after identification in order to meet the requirements of adequacy between vaccine strains and circulating epidemic influenza viruses.

Conclusion

The new SFM MDSS2N, free of any animal derived protein, supported cell growth of

- BHK-21/BRS cells in T- and spinner flasks,
- Vero cells in T-flasks and as microcarrier cultures in bioreactors, as well as
- MDCK cells in T-flasks and as microcarrier cultures in bioreactors as satisfactorily as MDSS2.

As for cell growth, both SFM were equivalent in supporting the production of

- rabies,
- polio, and
- influenza virus.

Altogether, the results are very promising for use of the SFM MDSS2N in a larger scale.

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